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TITLE: Unlocking Barriers to DNA Vaccine Immunogenicity: A Cross-Species Analysis of Cytosolic DNA Sensing in Skeletal Muscle Myocytes

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14. ABSTRACT

DNA vaccine technology holds great promise as a platform for developing vaccines against both emerging and established global pathogens. Despite this potential, significant challenges impede the capacity of DNA vaccines to prevent disease in humans. Foremost amongst these, is the gap between remarkable results obtained in pre-clinical mouse models and relatively modest immunogenicity observed in humans. The present work is testing the hypothesis that skeletal muscle myocytes sense cytosolic DNA and elaborate an inflammatory response to DNA vaccines and that species-specific differences in the cytosolic DNA sensing system bring about divergent inflammatory responses in human versus mouse skeletal muscle myocytes. We are pursuing the following specific aims: 1) characterize the inflammatory response elaborated by myocytes following the delivery of DNA to the cytosol, 2) define the components of the cytosolic DNA sensing system that are present in skeletal muscle myocytes and 3) ascertain which components of the myocyte cytosolic DNA sensing system are engaged upon delivery of DNA to the cytosol. We are utilizing molecular, biochemical and proteomic methods to analyze the consequences of DNA vaccine vector delivery into mouse and human myocyte-derived cell lines and primary cells. We anticipate that our efforts will produce important insights on cytosolic DNA sensors and provide a key to unlock DNA vaccine immunogenicity for humans.

15. SUBJECT TERMS
DNA vaccine, skeletal muscle myocytes, cytosolic DNA sensor

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Table of Contents

Page

Unlocking Barriers to DNA vaccine Immunogenicity: A Cross-Species Analysis of Cytosolic DNA Sensing in Skeletal Muscle Myocytes

1. INTRODUCTION:

We speculate that the skeletal muscle myocyte cytosolic DNA surveillance system can influence both the expression of and immune response to DNA vaccine antigens, thus impacting upon the immunogenicity of DNA vaccines. Moreover, we speculate that key differences in the function of DNA sensors/adaptors in mouse versus human myocytes contribute to the marked cross-species differences observed in DNA vaccine immunogenicity. We are therefore pursuing the hypothesis that skeletal muscle myocytes sense cytosolic DNA and elaborate the initial inflammatory response to DNA vaccines. We are also pursuing the secondary hypothesis that species-specific functional differences in the cytosolic DNA surveillance system bring about a divergent inflammatory response in human versus mouse skeletal muscle myocytes. Our ongoing project is focused on three specific aims including: 1) to define the components of the cytosolic DNA surveillance system that are present in human and mouse skeletal muscle myocytes, 2) to characterize the inflammatory response elaborated by human and mouse myocytes following delivery of DNA to the cytosol and 3) to ascertain which components of the human and mouse myocytes' cytosolic DNA surveillance systems are engaged and essential for the induction of inflammatory responses. This annual report describes our efforts to date in accomplishing these scientific objectives.

2. KEYWORDS:

DNA vaccine, cytosolic DNA sensor, skeletal muscle myocytes

3. ACCOMPLISHMENTS:

What were the major goals of the project?

Specific Aim 1: Define the components of the cytosolic DNA surveillance system that are present in human and mouse skeletal muscle myocytes

Major Task 1: Quantitative transcript analyses

Subtask 1A:
i) optimize culture and differentiation methods for **mouse myocyte cell lines** and evaluate phenotype of myocytes at different stages of differentiation using microscopy
ii) establish expression levels of **mouse** myogenic differentiation markers by RT-PCR and immunoblot
iii) establish expression levels of selected **mouse** cytosolic DNA sensors by RT-PCR

- Planned activity duration in SOW: 2015 Q4 – 2016 Q3

- Proportion of subtask completed: 100%
- Comment: This subtask has been completed using the mouse myocyte cell line model C2C12.

Subtask 1B:
i) optimize culture and differentiation methods for **mouse myocyte primary cells** and evaluate phenotype of myocytes at different stages of differentiation using microscopy
ii) establish expression levels of **mouse** myogenic differentiation markers by RT-PCR and immunoblot
iii) establish expression levels of selected **mouse** cytosolic DNA sensors by RT-PCR

- Planned activity duration in SOW: 2016 Q4 – 2017 Q1
- Proportion of subtask completed: 0%
- Proposed revision to activity duration in SOW: 2017 Q4 – 2018 Q1
- Comment: Further delay in onset of this activity reflects a decision to optimize assays and techniques using cell lines, prior to working with primary cells that are more difficult to obtain and maintain.

Subtask 1C:
i) optimize culture and differentiation methods for **human myocytes cell lines and primary cells** & evaluate phenotype of myocytes at different stages of differentiation using microscopy
ii) establish expression levels of **human** myogenic differentiation markers by RT-PCR and immunoblot
iii) establish expression levels of selected **human** cytosolic DNA sensors by RT-PCR

- Planned activity duration in SOW: 2016 Q4 – 2017 Q2
- Proportion of subtask completed: 15%
- Proposed revision to activity duration in SOW: 2017 Q4 – 2018 Q1
- Comment: Work with human cell lines and primary cells was delayed due to technical difficulty expanding the cell line and cost of reagents. We are currently working with human cell lines.

Subtask 2A:
i) configure a NanoString assay panel with candidate **mouse** cytosolic DNA sensors/adaptors and other relevant genes
ii) evaluate NanoString assay panel performance using **mouse** monocyte/macrophage cell lines

- Planned activity duration in SOW: 2017 Q1 – 2017 Q2
- Proportion of subtask completed: 0%
- Proposed revision to activity duration in SOW: 2017 Q4 – 2018 Q1
- Comment: This subtask has not been initiated. Given the relatively high cost of this technology, we will only proceed with its use in Q4 of 2016.

Subtask 2B:
i) configure a NanoString assay panel with candidate **human** cytosolic DNA sensors/adaptors and other relevant genes
ii) evaluate NanoString assay panel performance using **human** monocyte/macrophage cell lines

- Planned activity duration in SOW: 2017 Q2 – 2017 Q3
- Proportion of subtask completed: 0%
- Proposed revision to activity duration in SOW: 2017 Q4 – 2018 Q1
- Comment: This subtask has not been initiated. Given the relatively high cost of this technology, we will only proceed with its use in Q4 of 2016.

Subtask 3A:
i) evaluate mouse myocytes using configured NanoString panels
ii) validate gene expression for a small number of selected transcripts of interest queried by real-time quantitative RT-PCR

- Planned activity duration in SOW: 2017 Q2 – 2017 Q3
- Proportion of subtask completed: 0%
- Proposed revision to activity duration in SOW: 2017 Q4 – 2018 Q1
- Comment: This subtask has not been initiated. Given the relatively high cost of this technology, we will only proceed with its use in Q4 of 2016.

Subtask 3B:
i) evaluate human myocytes using configured NanoString panels
ii) validate gene expression for a small number of selected transcripts of interest queried by real-time quantitative RT-PCR

- Planned activity duration in SOW: 2017 Q3 – 2017 Q4
- Proportion of subtask completed: 0%
- Proposed revision to activity duration in SOW: 2017 Q4 – 2018 Q1
- Comment: This subtask has not been initiated. Given the relatively high cost of this technology, we will only proceed with its use in Q4 of 2016.

Milestone: We will determine which cytosolic sensors/adaptors and downstream effectors are expressed in human and mouse skeletal muscle myocytes.

- Milestone not achieved at this time

Major Task 2: Protein detection by standard immunoblotting

Subtask 1A:
i) analyze **mouse** cell lysates for selected cytosolic DNA sensors/adaptors using immunoblotting techniques (with and without immunoprecipitation methods to enrich proteins)

- Planned activity duration in SOW: 2016 Q1 – 2016 Q4
- Proportion of subtask completed: 70% 100%

Subtask 1B:
i) analyze **human** cell lysates for selected cytosolic DNA sensors/adaptors using immunoblotting techniques (with and without immunoprecipitation methods to enrich proteins)

- Planned activity duration in SOW: 2017 Q1 – 2017 Q3
- Proportion of subtask completed: 15%
- Proposed revision to activity duration in SOW: 2017 Q4 – 2018 Q1

Subtask 2A:
i) gauge expression and localization of selected **mouse** proteins by in-cell ELISA or quantitative immunofluorescence microscopy

- Planned activity duration in SOW: 2016 Q2 – 2017 Q1
- Proportion of subtask completed: 50%
- Proposed revision to activity duration in SOW: 2017 Q1 – 2018 Q1

Subtask 2B:
i) gauge expression and localization of selected **human** proteins by in-cell ELISA or quantitative immunofluorescence microscopy

- Planned activity duration in SOW: 2017 Q1 – 2017 Q3
- Proportion of subtask completed: 0%
- Proposed revision to activity duration in SOW: 2017 Q4 – 2018 Q1

Milestone: We will determine which cytosolic sensors/adaptors and downstream effectors are expressed in human and mouse skeletal muscle myocytes

- Milestone not achieved at this time

Specific Aim 2: Characterize the inflammatory response elaborated by human and mouse myocytes following delivery of DNA to the cytosol

Major Task 1: Transcription factor activation analysis

Subtask 1:
i) design and produce DNA vaccine vectors that incorporate a reporter protein and a viral antigen protein then verify by sequencing

ii) characterize expression using bioluminescence, immunofluorescence and immunoblotting methods

- Planned activity duration in SOW: 2015 Q4 – 2016 Q3
- Proportion of subtask completed: 100%

Subtask 2A:
i) use multiplex probe-capture technique and analysis on a Luminex platform to ascertain which **mouse** transcription factors are activated subsequent to entry of DNA

- Planned activity duration in SOW: 2016 Q4 – 2017 Q2
- Proportion of subtask completed: 0%
- Proposed revision to activity duration in SOW: 2017 Q4 – 2018 Q1

Subtask 2B:
i) use multiplex probe-capture technique and analysis on a Luminex platform to ascertain which **human** transcription factors are activated subsequent to entry of DNA

- Planned activity duration in SOW: 2017 Q1 – 2017 Q3
- Proportion of subtask completed: 0%
- Proposed revision to activity duration in SOW: 2017 Q4 – 2018 Q1

Subtask 3A:
i) validate **mouse** cell findings using chemiluminescent electrophoretic mobility shift assays (or DNA-binding ELISA technique in 96-well format)

- Planned activity duration in SOW: 2016 Q4 – 2017 Q3
- Proportion of subtask completed: 0%
- Proposed revision to activity duration in SOW: 2017 Q4 – 2018 Q1

Subtask 3B:
i) validate **human** cell findings using chemiluminescent electrophoretic mobility shift assays (or DNA-binding ELISA technique in 96-well format)

- Planned activity duration in SOW: 2017 Q2 – 2017 Q4
- Proportion of subtask completed: 0%
- Proposed revision to activity duration in SOW: 2017 Q4 – 2018 Q1

Subtask 4A:
i) evaluate key **mouse** cell transcription factors NFκB, IRF-3, and IRF-7 separately by immunoblotting of cellular extracts with phospho-specific antibodies

- Planned activity duration in SOW: 2016 Q4 – 2017 Q2

- Proportion of subtask completed: 50%
- Proposed revision to activity duration in SOW: 2017 Q1 – 2018 Q1

Subtask 4B:
i) evaluate key **human** cell transcription factors NFκB, IRF-3, and IRF-7 separately by immunoblotting of cellular extracts with phospho-specific antibodies

- Planned activity duration in SOW: 2017 Q1 – 2017 Q3
- Proportion of subtask completed: 0%
- Proposed revision to activity duration in SOW: 2017 Q4 – 2018 Q1

Milestone: We will ascertain which transcription factors are activated subsequent to entry of DNA into the cytosol, for both human and mouse skeletal muscle myocytes

- Milestone not achieved at this time

Major Task 2: Secretome analysis by multiplex cytokine capture

Subtask 1
As for Major Task 1

Subtask 2A:
i) detect and quantify **mouse** cytokines in supernatants using panel of cytokine-specific antibodies coupled to spectral signature-unique fluorescent beads with Luminex

- Planned activity duration in SOW: 2016 Q4 – 2017 Q2
- Proportion of subtask completed: 0%
- Proposed revision to activity duration in SOW: 2017 Q4 – 2018 Q1

Subtask 2B:
i) detect and quantify **human** cytokines in supernatants using panel of cytokine-specific antibodies coupled to spectral signature-unique fluorescent beads with Luminex

- Planned activity duration in SOW: 2017 Q2 – 2017 Q4
- Proportion of subtask completed: 0%
- Proposed revision to activity duration in SOW: 2017 Q4 – 2018 Q1

Subtask 3A:
i) validate specific mouse cell findings using immunoprecipitation with immunoblotting/ELISA
ii) further validate findings by utilizing immunofluorescence microscopy-based intracellular cytokine detection

- Planned activity duration in SOW: 2016 Q4 – 2017 Q2

- Proportion of subtask completed: 10%
- Proposed revision to activity duration in SOW: 2017 Q4 – 2018 Q1
- Comment: This subtask was initiated prior to initiating subtask 2A

Subtask 3B:
i) validate specific human cell findings using immunoprecipitation with immunoblotting/ELISA
ii) further validate findings by utilizing immunofluorescence microscopy-based intracellular cytokine detection

- Planned activity duration in SOW: 2017 Q3 – 2018 Q1
- Proportion of subtask completed: 0%
- Proposed revision to activity duration in SOW: 2017 Q4 – 2018 Q1

Milestone: We will determine which cytokines are secreted, by both human and mouse skeletal muscle myocytes, subsequent to entry of DNA into the cytosol

- Milestone not achieved

Specific Aim 3: Ascertain which components of the human and mouse myocytes' cytosolic DNA surveillance systems are engaged and essential for the induction of inflammatory responses

Major Task 1: "In vivo" protein-protein & DNA-protein cross-linking pull-down

Subtask 1A:
i) optimize membrane permeable chemical cross-linker method with **mouse** cells
ii) perform co-immunoprecipitation (co-IP) assays to pull-down proteins complexes formed in DNA exposed vs. non-exposed myocytes
iii) perform "time lapse" experiments

- Planned activity duration in SOW: 2016 Q4 – 2017 Q1
- Proportion of subtask completed: 10%
- Proposed revision to activity duration in SOW: 2017 Q1 – 2018 Q1

Subtask 1B:
i) optimize membrane permeable chemical cross-linker method with **human** cells
ii) perform co-immunoprecipitation (co-IP) assays to pull-down proteins complexes formed in DNA exposed vs. non-exposed myocytes
iii) perform "time lapse" experiments

- Planned activity duration in SOW: 2017 Q1 – 2017 Q2
- Proportion of subtask completed: 0%
- Proposed revision to activity duration in SOW: 2017 Q4 – 2018 Q1

Subtask 2A:
i) label plasmid DNA with photoactivatable biotin (e.g. Psoralen-PEG3-Biotin)
ii) perform DNA-protein complex pull-down method to pull-down DNA-protein complexes formed in DNA exposed vs. non-exposed **mouse** myocytes

- Planned activity duration in SOW: 2016 Q4 – 2017 Q2
- Proportion of subtask completed: 10%
- Proposed revision to activity duration in SOW: 2017 Q1 – 2018 Q1

Subtask 2B:
i) label plasmid DNA with photoactivatable biotin (e.g. Psoralen-PEG3-Biotin)
ii) perform DNA-protein complex pull-down method to pull-down DNA-protein complexes formed in DNA exposed vs. non-exposed **human** myocytes

- Planned activity duration in SOW: 2017 Q2 – 2017 Q3
- Proportion of subtask completed: 0%
- Proposed revision to activity duration in SOW: 2017 Q4 – 2018 Q1

Subtask 3A:
i) use "shotgun" proteomics methods (solubilized and denatured proteins are separated, subjected to tandem mass spectrometry (MS) and analyzed for identification) to determine the constituents of protein-protein and DNA-protein complexes derived from **mouse** cells

- Planned activity duration in SOW: 2017 Q2 – 2017 Q3
- Proportion of subtask completed: 0%
- Proposed revision to activity duration in SOW: 2017 Q4 – 2018 Q1

Subtask 3B:
i) use "shotgun" proteomics methods (solubilized and denatured proteins are separated, subjected to tandem mass spectrometry (MS) and analyzed for identification) to determine the constituents of protein-protein and DNA-protein complexes derived from human cells

- Planned activity duration in SOW: 2017 Q3 – 2017 Q4
- Proportion of subtask completed: 0%
- Proposed revision to activity duration in SOW: 2017 Q4 – 2018 Q1

Subtask 4A:
i) confirm specific mouse cell findings using tandem affinity tag purification method whereby candidate partners are co-expressed as 3X FLAG and Twin-StrepTag fusion proteins in mouse cells and subsequently subjected to bead-based affinity purification and immunoblotting or ELISA based detection

- Planned activity duration in SOW: 2017 Q2 – 2017 Q4

- Proportion of subtask completed: 0%
- Proposed revision to activity duration in SOW: 2017 Q4 – 2018 Q1

Subtask 4B:
i) confirm specific human cell findings using tandem affinity tag purification method whereby candidate partners are co-expressed as 3X FLAG and Twin-StrepTag fusion proteins in human cells and subsequently subjected to bead-based affinity purification and immunoblotting or ELISA based detection

- Planned activity duration in SOW: 2017 Q3 – 2018 Q1
- Proportion of subtask completed: 0%
- Proposed revision to activity duration in SOW: 2017 Q4 – 2018 Q1

Milestone Achieved: We will discover which cytosolic DNA sensors/adaptors and downstream effectors are operational in human and mouse skeletal muscle myocytes

- Milestone not achieved at this time

Major Task 2: Genome Engineering of Myocyte Cell Lines by CRISPR

Subtask 1A:
i) design and produce mammalian expression plasmids encoding the Cas9 protein and specially designed gRNAs (that directs the nuclease to complementary mouse genomic target sequences)
ii) confirm Cas9 and green fluorescent protein (GFP) reporter production by plasmids

- Planned activity duration in SOW: 2017 Q1 – 2017 Q2
- Proportion of subtask completed: 0%
- Proposed revision to activity duration in SOW: 2017 Q4 – 2018 Q1

Subtask 1B:
i) design and produce mammalian expression plasmids encoding the Cas9 protein and specially designed gRNAs (that directs the nuclease to complementary human genomic target sequences)
ii) confirm Cas9 and green fluorescent protein (GFP) reporter production by plasmids

- Planned activity duration in SOW: 2017 Q1 – 2017 Q2
- Proportion of subtask completed: 0%
- Proposed revision to activity duration in SOW: 2017 Q4 – 2018 Q1

Subtask 2A:
i) produce mouse myocyte cell lines that have undergone gene disruption via a technique named CRISPR
ii) confirm gene disruption and GFP expression

iii) select multiple individual clones characterized with quantitative gene expression

- Planned activity duration in SOW: 2017 Q1 – 2017 Q3
- Proportion of subtask completed: 0%
- Proposed revision to activity duration in SOW: 2017 Q4 – 2018 Q1

Subtask 2B:
i) produce human myocyte cell lines that have undergone gene disruption via a technique named CRISPR
ii) confirm gene disruption and GFP expression
iii) select multiple individual clones characterized with quantitative gene expression

- Planned activity duration in SOW: 2017 Q2 – 2017 Q4
- Proportion of subtask completed: 0%
- Proposed revision to activity duration in SOW: 2017 Q4 – 2018 Q1

Subtask 3A:
i) evaluate mouse cell line phenotype upon exposure to cytosolic DNA, as assessed by secretome and transcription factor analyses as described in Specific Aim 2, using cell lines produced with confirmed loss of the genes of interest

- Planned activity duration in SOW: 2017 Q3 – 2017 Q4
- Proportion of subtask completed: 0%
- Proposed revision to activity duration in SOW: 2017 Q4 – 2018 Q1

Subtask 3B:
i) evaluate human cell line phenotype upon exposure to cytosolic DNA, as assessed by secretome and transcription factor analyses as described in Specific Aim 2, using cell lines produced with confirmed loss of the genes of interest

- Planned activity duration in SOW: 2017 Q4 – 2018 Q1
- Proportion of subtask completed: 0%
- Proposed revision to activity duration in SOW: 2017 Q4 – 2018 Q1

Milestone: We will discover which cytosolic DNA sensors/adaptors and downstream effectors are essential for responding to cytosolic DNA in human and mouse skeletal muscle myocytes

- Milestone not achieved at this time

What was accomplished under these goals?

This is an ongoing project and the work described below supplements or refines the work presented in our last annual report. We are continuing to pursue all major objectives and specific objectives as delineated in our current SOW. We describe herein our ongoing success using the mouse myocyte cell line C2C12 as an initial model for cytosolic DNA sensing in myocytes and confirm some of our findings using other cell lines including the human cell line RD. Since our last report, we have gained a greater understanding of how myocyte levels of STING are modulated soon after entry of DNA into the cytosol following transfection. Specifically, we completed work aimed at understanding how STING is regulated. In addition, we have explored other components of the cytosolic DNA sensing pathway in myocytes. This has included cGAS, TBK-1 and IRF-3.

Further to last year's annual report, the following additional work have been completed for the 12-month period ending September 9th, 2017.:

1) Our preliminary data suggested that STING abundance diminished after introduction of DNA to the cytosol. We have since carried out multiple experiments that reproducibly show that indeed the quantity of STING protein in myocytes diminishes markedly after transfection with plasmid DNA. This was shown to be the case in both myoblasts and myotubes. In most of our experiments, this diminution in STING quantity began as early as 2 hours after transfection, then persisted for at least 48 hours after transfection (**Figure 1A**). This implied that STING abundance was regulated by engagement of the cytosolic DNA sensing pathway in myocytes. To explore the possibility that this phenomenon was triggered via the upstream cytosolic DNA sensor cGAS, we performed experiments using the murine cGAS ligand DMXAA. These experiments revealed a similar phenomenon (**Figure 2B**) and supported a model whereby STING is activated downstream of cGAS engaging DNA (or a chemical ligand). It was likely that in this context STING was being rapidly degraded but we sought to exclude the possibility that any regulation at the transcriptional level was occurring at early time points after transfection. Repeated experiments reproducibly showed that no significant diminution of STING at the transcript level occurred at time points that would explain the loss of STING early post transfection (**Figure 1C**).

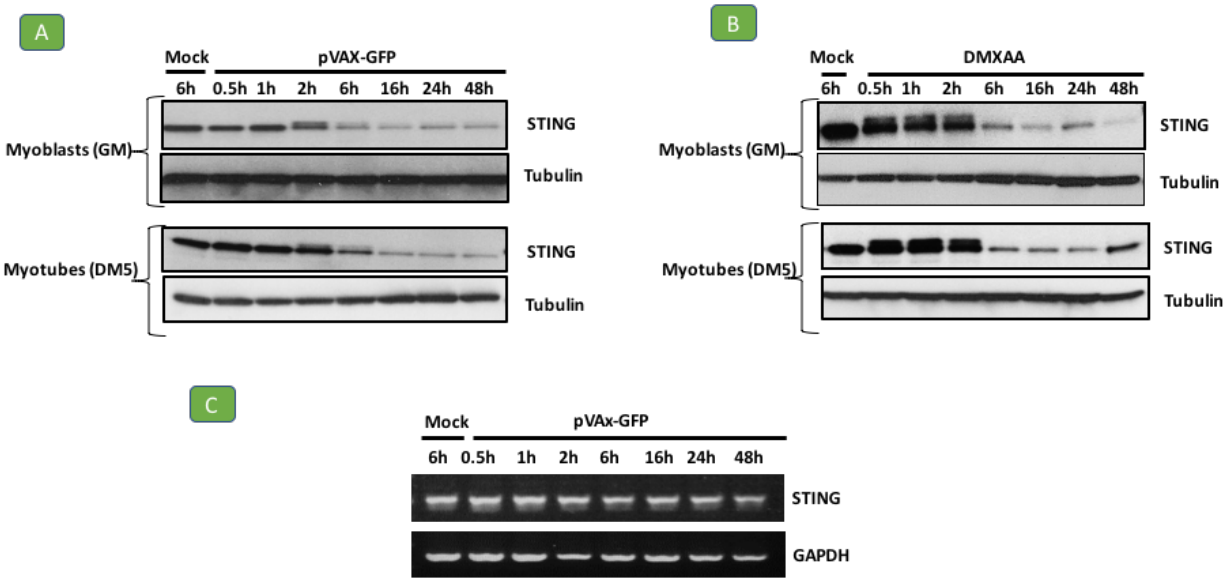

Figure 1. (A) STING abundance diminishes between 2h and 6h after transfection of C2C12 cells with pVAX-GFP. Protein lysates from myoblast (GM) or myotubes (DM5) were collected 6h after mock transfection without DNA (Mock) or 0.5h, 1h, 2h, 6h, 16h, 24h, 48h after transfection with pVAX-GFP and were blotted with anti-STING antibody. Blotting with anti-tubulin antibody was used as a loading control. **(B) STING abundance diminishes between 2h and 6h after incubation of C2C12 cells with DMXAA.** Protein lysates from myoblast (GM) or myotubes (DM5) were collected 6h after mock incubation with DMSO (Mock) or 0.5h, 1h, 2h, 6h, 16h, 24h, 48h after incubation with DMXAA and were blotted with anti-STING antibody. Blotting with anti-tubulin antibody was used as a loading control. **(C) Loss of STING does not occur at the RNA level.** RT-PCR analysis was performed using total RNA extracts from myoblasts collected 6h after mock transfection without DNA (Mock) or 0.5h, 1h, 2h, 6h, 16h, 24h, 48h after transfection with pVAX-GFP. Mouse-STING specific primers were used to amplify STING and Mouse-GAPDH primers were used for GAPDH as control.

2) Based on accumulating published data, the cGAS-STING pathway appears to be the major cytosolic DNA sensing pathway operational in immune cells such as macrophages. After engagement of dsDNA by cGAS, cyclic dinucleotides are synthesized and act as second messengers to induce activation of STING. STING subsequently interacts with TBK-1 and this interaction results in phosphorylation of both STING and TBK-1. Downstream transcription factors, notably IRF-3, are subsequently phosphorylated and translocate to the nucleus to trigger gene expression. Considering this information, we sought to ascertain whether cGAS and TBK-1 were also expressed in myocytes. Indeed, both proteins were detectable in C2C12 myoblasts (**Figure 2A and 2B**). After DNA transfection or DMXAA treatment, the abundance of neither cGAS nor TBK-1 appeared to vary significantly over time however phospho-TBK-1 appeared to increase in abundance then diminish back to baseline (**Figure 2A and 2B**). Interestingly, DMXAA appeared to induce an earlier accumulation of phospho-TBK-1, presumably due to more rapid interaction or more complete saturation of cGAS. In contrast to C2C12, the murine macrophage cell line RAW 264.7 appeared to maintain steady levels of levels of STING and phospho-TBK-1 despite DNA transfection (**Figure 2C**). This observation implies that RAW 264.7 are in a constitutively activated state whereas C2C12 tightly regulate the cytosolic DNA sensing pathway. We attempted to prove that this phenomenon also differentially induced transcription factor activation, particularly at the level of IRF-3,

however in our hands detection of phospho-IRF-3 have been challenging (see **PROBLEMS SECTION** below). Activation of the transcription factor NFκB is also ongoing.

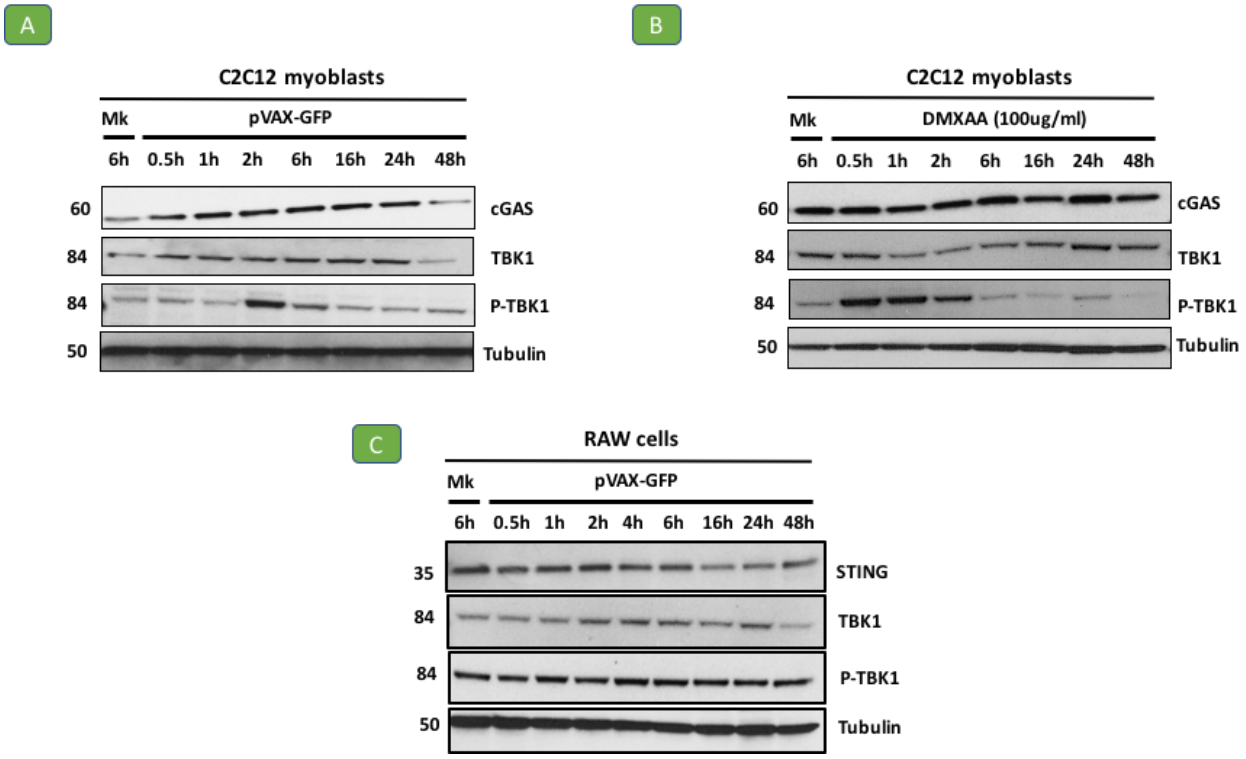

Figure 2. (A and B) Presence of other components of the cytosolic DNA sensing pathway in C2C12. C2C12 cells were (A) transfected with pVAX-GFP or (B) treated with DMXAA. Protein lysates from myoblasts were collected (A) 6h after mock transfection without DNA (Mk) and 0.5h, 1h, 2h, 6h, 16h, 24h, 48h after transfection with a plasmid or (B) 6h after treatment with vehicle (Mk) and 0.5h, 1h, 2h, 6h, 16h, 24h, 48h after treatment with DMXAA. The lysates were blotted with anti-STING, anti-cGAS, anti-TBK1, anti-phospho-TBK1 antibodies. Blotting with anti-tubulin antibody was used as a loading control. **(C) Presence of components of the cytosolic DNA sensing pathway in RAW 264.7.** Protein lysates were collected 6h after mock transfection without DNA or 0.5h, 1h, 2h, 4h, 6h, 16h, 24h, 48h after transfection with pVAX-GFP. The lysates were blotted with anti-STING, anti-cGAS, anti-TBK1, anti-phospho-TBK1 antibodies. Blotting with anti-tubulin or anti-GAPDH antibodies was used as a loading control.

3) We noted that prior to a diminution in abundance on immunoblots, there appeared to be a mobility shift of STING to a higher molecular weight form (Figure 1A & 1B). Given STING activation in other cell models involves phosphorylation, we surmised that this mobility shift was caused by phosphorylation. No STING phosphorylation site-specific antibodies are presently available thus we collected myocyte lysates at different time points after DNA transfection then treated lysates them with lambda phophorylase and subjected them to immunoblot analysis. Indeed, dephosphorylation of lysates caused the higher molecular weight STING isoform to disappear (**Figure 3A**), supporting the idea that phosphorylation of STING occurred after DNA transfection and preceded degradation. Based on published data in immune cells, we further postulated that inhibiting trafficking of STING from its location in the endoplasmic reticulum (ER) to the

Golgi might abrogate phosphorylation and degradation. To this end we used pre-treatment of cells with brefeldin A to block ER to Golgi trafficking. Pre-treatment with BFA blocked production of higher molecular weight STING phosphorylated isoforms and abrogated the loss of STING (**Figure 3B**) induced by treatment with DMXAA. Interestingly, whereas phosphorylated forms of TBK-1 quickly accumulated after treatment with DMXAA, pre-treatment with BFA also blocked production of phospho-TBK-1 (**Figure 3B**), implying that STING and/or TBK-1 trafficking to the Golgi was necessary for TBK-1 phosphorylation.

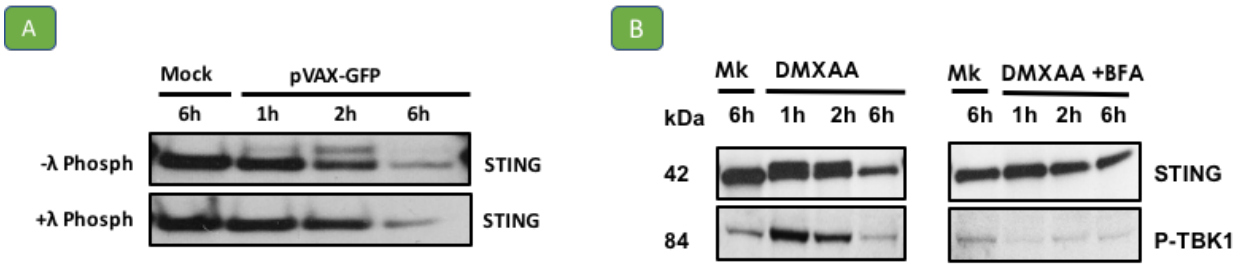

Figure 3. (A) STING is phosphorylated prior to degradation. Protein lysates from myoblasts were collected 6h after mock transfection without DNA (Mock) or 1h, 2h and 6h after transfection with a plasmid. The lysates were treated with λ Phosphatase (100U) and were blotted with anti-STING antibody. **(B) Phosphorylation and degradation of STING and phosphorylation of TBK-1 is abolished after treatment with Brefeldin A (BFA).** C2C12 myoblasts were pre-treated with brefeldin A (BFA) or vehicle. Protein lysates were collected 6h after mock treatment with vehicle (Mock) or 1h, 2h and 6h after incubation with DMXAA. The lysates were blotted with anti-STING and anti-phospho-TBK1 antibodies.

4) Loss of STING at the protein level soon after DNA transfection or treatment with DMXAA suggested that STING was being rapidly degraded. We postulated that one of two potential degradation pathways might be implicated in STING regulation, namely proteasomal degradation or lysosomal degradation via autophagy. To assess for the role of proteasomal degradation we performed experiments using pre-treatment with MG132, an inhibitor of the proteasome pathway. We found that this pre-treatment partially rescued STING from degradation (**Figure 4A**). To probe whether lysosomal degradation was being induced by an autophagy-related process we performed experiments using pre-treatment with agents that induce alkalinisation of the lysosome. Pre-treatment with either agent completely rescued STING from degradation and appeared to delay but not abrogate the accumulation of phospho-TBK-1 (**Figure 4B and 4C**). The results of these experiments implied that lysosomal and perhaps proteasomal degradation are important events in STING regulation in myocytes.

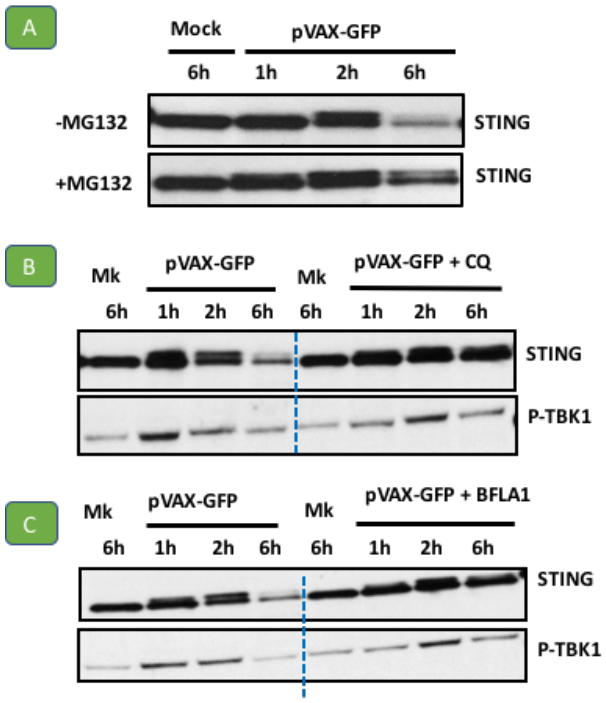

Figure 4. (A) STING is partially rescued from degradation by pre-treatment with an inhibitor of the proteasome pathway. C2C12 myoblasts were pre-treated with MG132 or with vehicle. Protein lysates from myoblasts were collected 6h after mock transfection without DNA (Mock) or 1h, 2h and 6h after transfection with plasmid and were blotted with anti-STING antibody. **(B and C) STING is rescued from degradation by pre-treatment with agents that induce lysosomal alkalinisation.** C2C12 myoblasts were pre-treated with **chloroquine (**CQ) or **bafilomycin A1 (**BFLA1) or corresponding vehicle. Protein lysates were collected 6h after mock transfection without DNA (Mock) or 1h, 2h and 6h after transfection with the plasmid. The lysates were blotted with anti-STING and anti-phospho-TBK1 antibodies.

5) STING appears to exist as a dimer that spans the ER membrane but engagement of its inter-dimer interface by cyclic dinucleotides produces a conformational change that allows it to interact with other proteins such as TBK-1. We have initiated experiments using chemical cross-linkers to visualize and capture STING and STING binding partners in a stabilized form after STING activation. Thus far, we have conducted experiments with cell-permeable, thiol-cleavable cross-linkers. For example, dithiobis[succinimidyl propionate] (DSP) reacts with primary amines and has a spacer-arm of 12 Å forming crosslinks between lysine residues of interacting proteins. These bonds can be cleaved by using a reducing agent. To date this “in cell cross-linking” technique has allowed us to visualize small quantities of STING dimers that are only generated after DNA transfection of myocytes (**Figure 5**). Using immunoprecipitation techniques and additional chemical cross-linkers, we are attempting to optimize visualization and capture these STING dimers and other proteins interacting with STING. Successful optimization of this technique would allow us to identify STING interacting partners using traditional immunoblotting techniques and MALDI-TOF based proteomic analyses.

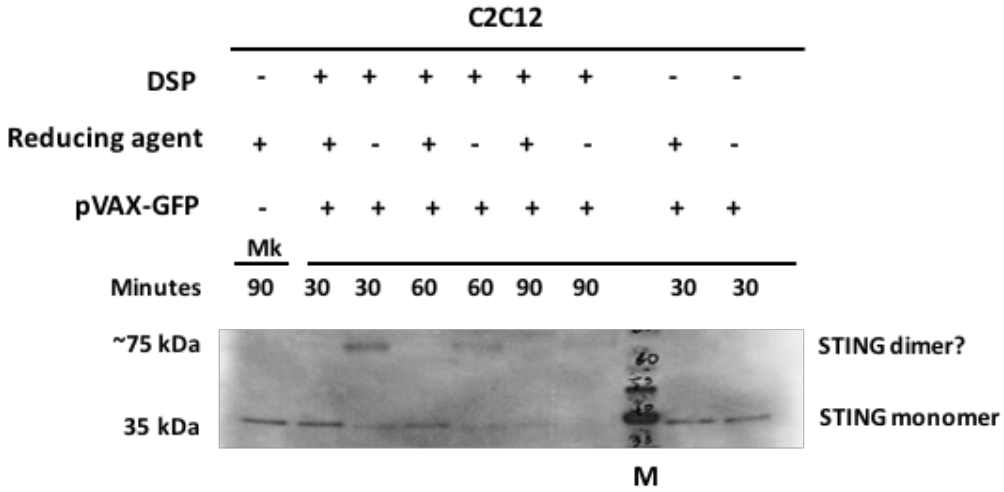

Figure 5. DSP crosslinking after transfection of C2C12 cells with pVAX-GFP. DSP was added to the cells 30 min, 60 min or 90 min after transfection. The protein lysates were processed with or without reducing agent and were blotted with anti-STING antibody. Blotting with anti-tubulin antibody was used as a loading control.

6) Previously published data on cytosolic DNA sensing in immune cells such as macrophages has revealed that this pathway can induce the expression of type I interferons and other cytokines. We therefore wanted to ascertain whether myocytes can be induced to express IFNβ1 or IL6, two factors that can be robustly induced in macrophages upon triggering the cytosolic DNA sensing pathway. To this end we compared the myocyte cell line, C2C12 to the macrophage cell line RAW 264.7. C2C12 expressed very low levels of IL6 but no IFNβ1 (**Figure 6A**). DNA transfection rapidly induced greater expression of the expression of IL6 and induced expression of IFNβ1 which only became detectable after a prolonged interval post-transfection (**Figure 6A**). Conversely, relatively abundant transcript levels of IFNβ1 and IL6 were present in RAW 264.7 before DNA transfection (**Figure 6B**). Some induction of IFNβ1 expression did occur after DNA transfection however (**Figure 6B**).

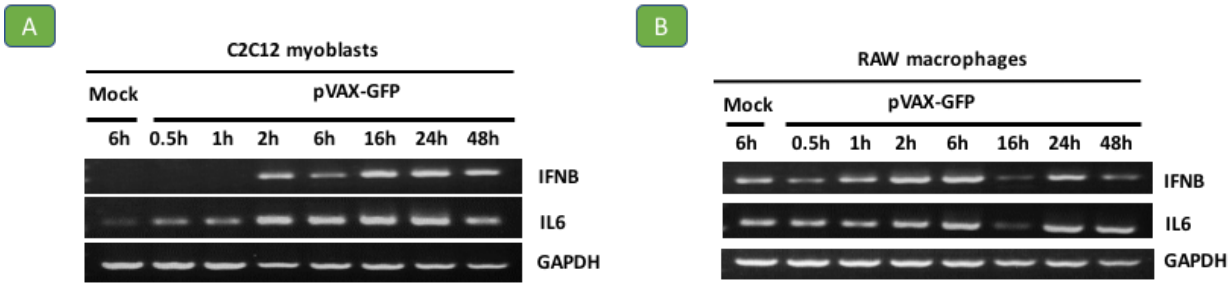

Figure 6. DNA transfection of C2C12 triggers IFNβ1 and IL6 expression that appears to coincide with STING activation. (A) IFNβ1 and IL6 expression in C2C12 cells. Two-step RT-PCR analysis was performed using total RNA extracts from myoblasts collected 6h after mock transfection without DNA (Mock) or 0.5h, 1h, 2h, 6h, 16h, 24h, 48h after transfection with pVAX-GFP. Mouse-IFNβ1 and mouse-IL6 specific primers were used to amplify IFNβ1 and IL6 respectively. Mouse-GAPDH primers were used to amplify GAPDH as an internal control. **(B) IFNβ1 and IL6 expression in RAW 264.7.** Two-step RT-PCR analysis was performed using total RNA extracts from macrophages cells collected 6h after mock transfection without DNA (Mock) or 0.5h, 1h, 2h, 6h, 16h, 24h, 48h after transfection with pVAX-GFP. Mouse-IFNβ1 and mouse-IL6 specific primers were used to amplify IFNβ1 and IL6 respectively. Mouse-GAPDH primers were used to amplify GAPDH as an internal control.

7) The production and secretion of IFNβ1 by skeletal muscle myocytes has not been previously described. In our last annual report, we briefly described unsuccessful attempts to detect IFNβ1 in skeletal muscle myocytes using ELISA-based techniques. After extensive efforts (see **PROBLEMS SECTION** below), we developed an enhanced detection strategy combining three different but complementary approaches. Using brefeldin A (BFA) added after DNA transfection, we blocked protein secretion (by blocking ER to Golgi transit) after induction of cytokine expression. This would be expected to allow accumulation of cytokines intracellularly, potentially enhancing their detection. Secondly, as murine IFNβ1 is known to have several glycoforms we used PNGase F to deglycosylate proteins in cell lysates thought to contain IFNβ1 to enhance their visualization on immunoblots. Finally, we attempted to enhance detection of small quantities of IFNβ1 by using used immunoprecipitation methods. The combination of BFA treatment of DNA transfected cells and deglycosylation of subsequent cell lysates prior to immunoblotting did not allow us to detect IFNβ1 at the protein level in C2C12, although IFNβ1 was readily detected in RAW 264.7 (**Figure 7A**). We then carried out a similar procedure with the addition of immunoprecipitation of IFNβ1 prior to deglycosylation and immunoblotting. This appeared to yield a protein of a molecular weight consistent with IFNβ1 (**Figure 7B**). These findings require further confirmation with additional control experiments.

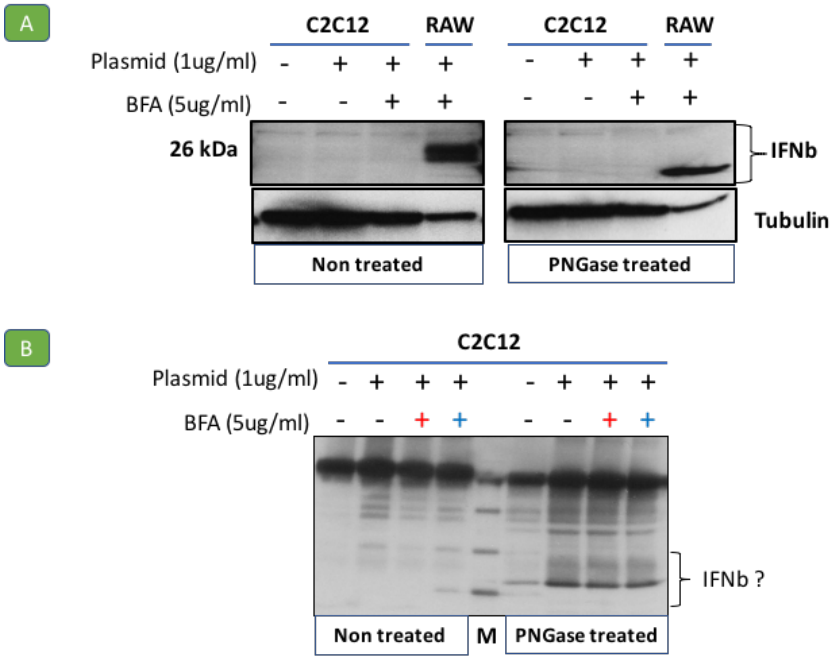

Figure 7. (A) IFNβ1 protein is not detected in C2C12 cells despite treatment of cells with brefeldin A and deglycosylation of cell lysates. C2C12 and RAW cells were transfected with pVAX-GFP plasmid. Brefeldin A was added 1h after transfection to block secretion and allow intracellular accumulation of IFNβ1. The lysates were also treated with PNGase F to deglycosylate IFNβ1 and were blotted with anti-IFNβ1 antibody (D2J1D). **(B) Immunoprecipitation of IFNβ1 in C2C12 appears to allow visualization.** C2C12 were transfected with pVAX-GFP plasmid. Brefeldin A was added immediately after transfection (red plus sign) or 2h post transfection (blue plus sign). Anti-IFNβ1 protein A purified antibody was added to cell lysates followed by the addition of Protein A beads. After an overnight incubation and several washes, the proteins attached to the beads were deglycosylated with PNGase F and the eluted proteins were blotted with anti-IFNβ1 antibody (D2J1D).

8) In immune cells such as macrophages, STING appears to be localized to the ER. After activation induced by the entry of DNA into the cytosol, STING traffics to the Golgi and an ill-defined peri-nuclear compartment. Aiming to define the localization and trafficking of STING in skeletal muscle myocytes after DNA transfection or treatment with DMXAA, we first sought to confirm its presence in the myocyte cytoplasm using immunofluorescence microscopy. This proved to be unexpectedly difficult (see **PROBLEMS SECTION** below). Our efforts eventually confirmed cytosolic localization using different STING-specific antibodies (**Figure 8**). Efforts to define STING trafficking after DNA transfection or DMXAA treatment using immunofluorescence have thus far proved difficult (see **PROBLEMS SECTION** below). This work is ongoing as such information will be essential to understanding the cytosolic DNA sensing pathway in skeletal muscle myocytes.

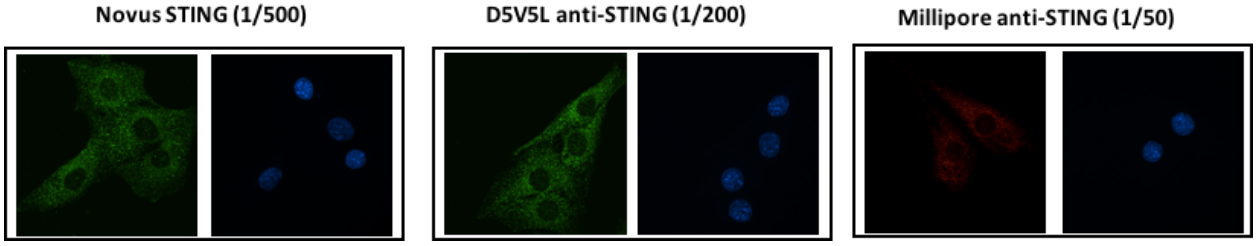

Figure 8. Localization of STING in C2C12 cells using immunofluorescence microscopy. Three different antibodies were used to determine the subcellular location of STING in C2C12 cells. The cells were incubated with respectively 1:500 anti STING (NovusBio), 1:200 anti-STING (D5V5L) and 1:50 anti-STING (Millipore) followed by an incubation with donkey anti-rabbit Alexa 488 conjugated antibody or donkey anti-mouse Alexa 594 conjugated antibody.

9) We have also begun work aimed at understanding STING regulation in human skeletal muscle myocytes. Our present efforts have focused on the cell line RD. Interestingly, this cell line maintains steady levels of STING despite DNA transfection however phosphorylation of TBK-1 is induced by DNA and persists for a prolonged period (**Figure 9**). This difference between the human RD and mouse C2C12 models, points to the possibility that human and mouse skeletal muscle myocytes regulate the cytosolic DNA signaling pathway using distinct mechanisms. These findings require further investigation.

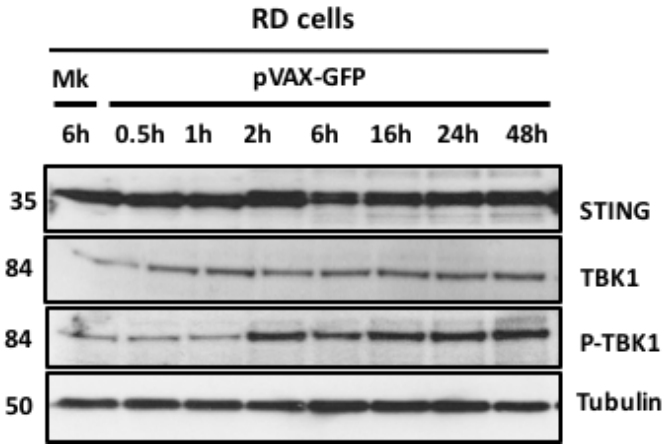

Figure 9. Presence of STING and TBK-1 in the human skeletal muscle-derived myocyte cell line RD. Protein lysates were collected 6h after mock transfection without DNA or 0.5h, 1h, 2h, 4h, 6h, 16h, 24h, 48h after transfection with pVAX-GFP. The lysates were blotted with anti-STING, anti-cGAS, anti-TBK1, anti-phospho-TBK1 antibodies. Blotting with anti-tubulin or anti-GAPDH antibodies was used as a loading control.

What opportunities for training and professional development has the project provided?

The research personnel working on this project have continued to receive mentorship and additional training from the principal investigator regarding general experimental design, DNA expression vector design, tissue culture, immunoblotting techniques, primer design and RT-PCR techniques. As compared to our prior annual report, much more extensive training in fluorescent microscopy techniques has been provided through the investigators' core facility. Professional development activities in the form of weekly scientific axis-specific seminars, postdoctoral fellow seminars, a biostatistics seminar series have also been provided to research personnel.

How were the results disseminated to communities of interest?

Our preliminary results were presented at our institution's annual scientific retreat: Edaye S, Gheorghe R and **Zaharatos GJ**. Cytosolic DNA sensing in skeletal muscle-derived myocytes. Poster Abstract. Eighth Annual Lady Davis Institute Scientific Retreat, May 12th, 2017. Montreal, Quebec, Canada. ***Please see the appendix for a copy of our poster abstract.***

We have also submitted an abstract for consideration to the Keystone Symposia's Emerging Technologies in Vaccine Discovery and Development (J5) meeting to be held January 28 - February 1, 2018 in Banff, Alberta, Canada. The abstract is entitled "Are myocytes actors in DNA vaccine immunogenicity? In vitro studies of cytosolic DNA sensing in skeletal muscle myocytes". ***Please see the appendix for a copy of our submitted abstract.***

A manuscript describing our findings in mouse cells is presently being prepared. We expect to be able to submit this manuscript some time in November 2017.

What do you plan to do during the next reporting period to accomplish the goals?

We expect that the targeted approach we have taken thus far will guide subsequent work and allow us to use our resources more effectively in the last six months of the project. More advanced but costly techniques, likely to generate a large amount of data, are thus only now being used. In this manner, we hope to accomplish most of our goals while staying within the confines of our budget.

`

4. **IMPACT:**

What was the impact on the development of the principal discipline(s) of the project?

Nothing to Report

What was the impact on other disciplines?

Nothing to Report

What was the impact on technology transfer?

Nothing to Report

What was the impact on society beyond science and technology?

Nothing to Report

5. **CHANGES/PROBLEMS:**

Changes in approach and reasons for change

No changes in approach are anticipated. There are no methodology changes to report regarding the major goals of the project or scientific objectives. Given delays in completing some tasks due to difficulties or challenges with certain techniques, a delay or adjustment in the onset and duration of some activities is proposed in the form of a revised SOW (see **APPENDIX**).

Actual or anticipated problems or delays and actions or plans to resolve them

We alluded to several methodologic or technical challenges in the section above describing our accomplishments. These challenges prolonged effort and expenditure on certain aspects of the project and delayed the onset of certain tasks. Major problems that resulted in delays in attaining some of our goals included the following:

1) Significant expenditure of time and effort and reagents was made attempting to select and optimize techniques to detect components of the cytosolic DNA sensing signaling pathway in myocytes. While we eventually optimized detection of STING, cGAS, TBK-1 and phosphorylated TBK-1 we continue to have great difficulty accurately detecting the transcription factor IRF-3 and its phosphorylated form (phospho-IRF-3). We are presently working with new antibodies and will also use native non-denaturing gel electrophoresis to detect dimerized IRF-3 as a surrogate for its activated form.

2) Significant expenditure of time and effort and reagents was made attempting to select and optimize techniques to detect IFNβ1 protein in myocytes. As described above, we are in the process of optimizing a new technique to enhance IFNβ1detection. It is possible however that unlike immune cells such as macrophages, IFNβ1 is simply not expressed in sufficient quantity or alternatively is too quickly degraded to allow detection but standard methods. Femtomolar level detection using mass spectrometry methods may be required in this case.
3) The most significant expenditure of time and effort and reagents was made attempting to optimize STING localization and trafficking using immunofluorescence microscopy methods. The first challenge we faced was the mislocalization of STING induced by various detergents we used for myocyte permeabilization after fixation. After extensive testing, we have now solved this by using low concentrations of saponin. Concurrently, finding appropriate antibodies for immunofluorescence (unlike immunoblotting) was highly problematic and in fact only a handful of antibodies appeared adequate. Our efforts to find the ideal antibody are ongoing as we believe that we are still detecting significant background fluorescence with all the antibodies we have evaluated. This background has hindered our attempts to visualize STING trafficking after DNA enters the myocyte cytosol. Although we continue to optimize this technique, we may have to pursue an alternate approach such as generating myocyte cell lines that express epitope tagged or fluorescent protein tagged STING.

Changes that had a significant impact on expenditures

Given the problems described above, greater than expected time, effort and expenditures were made on this project to date. This work involved many experiments analyzing cell lysates for cytosolic DNA sensors/adaptors using immunoblotting techniques as well as extensive work optimizing immunofluorescence microscopy. Accordingly, less was spent on using advanced but more costly techniques (e.g. Luminex and NanoString methods). We did however attempt to keep expenditures in check by initially working with cell lines rather than primary cells, as the latter system requires a greater use of resources. However, other than shifting some expenditures to later in the project timeline, we do not expect any overall impact on total expenditures required to complete the project.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Nothing to report

Significant changes in use or care of human subjects

Not applicable

Significant changes in use or care of vertebrate animals.

Nothing to report

Significant changes in use of biohazards and/or select agents

Nothing to report

6. **PRODUCTS:**

Nothing to Report

7. **PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS**

What individuals have worked on the project?

Name:	**Dr. Gerasimos J. Zaharatos**
Project Role:	Principal Investigator
Researcher Identifier:	not registered
Nearest person month worked:	12
Contribution to Project:	• Provided training to personnel (postdoc and research technician) • Supervised all work • Designed overall research strategy • Guided experimental design • Assisted in analysis of data
Funding Support:	No other support

Name:	**Dr. Sonia Edaye**
Project Role:	Postdoctoral Fellow
Researcher Identifier:	not registered
Nearest person month worked:	12

Contribution to Project:	• Provided training to personnel (research technician) • Supervised work of research technician • Assisted in experimental design • Executed experiments • Analyzed data
Funding Support:	No other support

Name:	**Ms. Romina Gheorghe**
Project Role:	Research technician
Researcher Identifier:	not registered
Nearest person month worked:	12
Contribution to Project:	• Executed experiments • Assisted in analysis of data
Funding Support:	No other support

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to Report

What other organizations were involved as partners?

Nothing to Report

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: Not applicable

QUAD CHARTS: Not applicable

9. **APPENDICES:**

1) Proposed SOW revision pertaining to end dates for certain subtasks
2) Poster abstract presented at Eighth Annual Lady Davis Institute Scientific Retreat
3) Abstract submitted to Keystone Symposia's Emerging Technologies in Vaccine Discovery and Development (J5) meeting

STATEMENT OF WORK – REVISED OCTOBER 9, 2017.
START DATE – SEPTEMBER 10, 2015.

JEWISH GENERAL HOSPITAL,
3755 COTE-STE-CATHERINE ROAD, ROOM E-0057
MONTREAL, QUEBEC, CANADA, H3T 1E2

PI: **GERASIMOS JERRY ZAHARATOS, MD**

Title of Proposal: Unlocking Barriers to DNA Vaccine Immunogenicity: A Cross-Species Analysis of Cytosolic DNA Sensing in Skeletal Muscle Myocytes

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Research-Specific Tasks:

Specific Aim 1: Define the components of the cytosolic DNA surveillance system that are present in human and mouse skeletal muscle myocytes	**Year & Quarter (start to finish)**
Major Task 1: *Quantitative transcript analyses*	
Subtask 1A: i) optimize culture and differentiation methods for **mouse myocyte cell lines** and evaluate phenotype of myocytes at different stages of differentiation using microscopy ii) establish expression levels of **mouse** myogenic differentiation markers by RT-PCR and immunoblot iii) establish expression levels of selected **mouse** cytosolic DNA sensors by RT-PCR	**2015 Q4 – 2016 Q3**
Subtask 1B: i) optimize culture and differentiation methods for **mouse myocyte primary cells** and evaluate phenotype of myocytes at different stages of differentiation using microscopy ii) establish expression levels of **mouse** myogenic differentiation markers by RT-PCR and immunoblot iii) establish expression levels of selected **mouse** cytosolic DNA sensors by RT-PCR	**2017 Q4 – 2018 Q1**
Subtask 1C: i) optimize culture and differentiation methods for **human myocytes cell lines and primary cells** & evaluate phenotype of myocytes at different stages of differentiation using microscopy ii) establish expression levels of **human** myogenic differentiation markers by RT-PCR and immunoblot iii) establish expression levels of selected **human** cytosolic DNA sensors by RT-PCR	**2017 Q4 – 2018 Q1**

Subtask 2A: i) configure a NanoString assay panel with candidate **mouse** cytosolic DNA sensors/adaptors and other relevant genes ii) evaluate NanoString assay panel performance using **mouse** monocyte/macrophage cell lines	**2017 Q4 – 2018 Q1**
Subtask 2B: i) configure a NanoString assay panel with candidate **human** cytosolic DNA sensors/adaptors and other relevant genes ii) evaluate NanoString assay panel performance using **human** monocyte/macrophage cell lines	**2017 Q4 – 2018 Q1**
Subtask 3A: i) evaluate **mouse** myocytes using configured NanoString panels ii) validate gene expression for a small number of selected transcripts of interest queried by real-time quantitative RT-PCR	**2017 Q4 – 2018 Q1**
Subtask 3B: i) evaluate **human** myocytes using configured NanoString panels ii) validate gene expression for a small number of selected transcripts of interest queried by real-time quantitative RT-PCR	**2017 Q4 – 2018 Q1**
Milestone Achieved: ***We will determine which cytosolic sensors/adaptors and downstream effectors are expressed in human and mouse skeletal muscle myocytes***	
Major Task 2: ***Protein detection by standard immunoblotting***	
Subtask 1A: i) analyze **mouse** cell lysates for selected cytosolic DNA sensors/adaptors using immunoblotting techniques (with and without immunoprecipitation methods to enrich proteins)	**2016 Q1 – 2016 Q4**
Subtask 1B: i) analyze **human** cell lysates for selected cytosolic DNA sensors/adaptors using immunoblotting techniques (with and without immunoprecipitation methods to enrich proteins)	**2017 Q4 – 2018 Q1**
Subtask 2A: i) gauge expression and localization of selected **mouse** proteins by in-cell ELISA or quantitative immunofluorescence microscopy	**2017 Q1 – 2018 Q1**
Subtask 2B: i) gauge expression and localization of selected **human** proteins by in-cell ELISA or quantitative immunofluorescence microscopy	**2017 Q4 – 2018 Q1**

Milestone Achieved: ***We will determine which cytosolic sensors/adaptors and downstream effectors are expressed in human and mouse skeletal muscle myocytes***	
Specific Aim 2: Characterize the inflammatory response elaborated by human and mouse myocytes following delivery of DNA to the cytosol	**Year & Quarter (start to finish)**
Major Task 1: *Transcription factor activation analysis*	
Subtask 1: i) design and produce DNA vaccine vectors that incorporate a reporter protein and a viral antigen protein then verify by sequencing ii) characterize expression using bioluminescence, immunofluorescence and immunoblotting methods	**2015 Q4 – 2016 Q3**
Subtask 2A: i) use multiplex probe-capture technique and analysis on a Luminex platform to ascertain which **mouse** transcription factors are activated subsequent to entry of DNA	**2017 Q4 – 2018 Q1**
Subtask 2B: i) use multiplex probe-capture technique and analysis on a Luminex platform to ascertain which **human** transcription factors are activated subsequent to entry of DNA	**2017 Q4 – 2018 Q1**
Subtask 3A: i) validate **mouse** cell findings using chemiluminescent electrophoretic mobility shift assays (or DNA-binding ELISA technique in 96-well format)	**2017 Q4 – 2018 Q1**
Subtask 3B: i) validate **human** cell findings using chemiluminescent electrophoretic mobility shift assays (or DNA-binding ELISA technique in 96-well format)	**2017 Q4 – 2018 Q1**
Subtask 4A: i) evaluate key **mouse** cell transcription factors NFκB, IRF-3, and IRF-7 separately by immunoblotting of cellular extracts with phospho-specific antibodies	**2017 Q1 – 2018 Q1**
Subtask 4B: i) evaluate key **human** cell transcription factors NFκB, IRF-3, and IRF-7 separately by immunoblotting of cellular extracts with phospho-specific antibodies	**2017 Q4 – 2018 Q1**
Milestone Achieved: ***We will ascertain which transcription factors are activated subsequent to entry of DNA into the cytosol, for both human and mouse skeletal muscle myocytes***	

Major Task 2: *Secretome analysis by multiplex cytokine capture*	
Subtask 1 As for Major Task 1	**2015 Q4 – 2016 Q3**
Subtask 2A: i) detect and quantify **mouse** cytokines in supernatants using panel of cytokine-specific antibodies coupled to spectral signature-unique fluorescent beads with Luminex	**2017 Q4 – 2018 Q1**
Subtask 2B: i) detect and quantify **human** cytokines in supernatants using panel of cytokine-specific antibodies coupled to spectral signature-unique fluorescent beads with Luminex	**2017 Q4 – 2018 Q1**
Subtask 3A: i) validate specific **mouse** cell findings using immunoprecipitation with immunoblotting/ELISA ii) further validate findings by utilizing immunofluorescence microscopy-based intracellular cytokine detection	**2017 Q4 – 2018 Q1**
Subtask 3B: i) validate specific **human** cell findings using immunoprecipitation with immunoblotting/ELISA ii) further validate findings by utilizing immunofluorescence microscopy-based intracellular cytokine detection	**2017 Q4 – 2018 Q1**
***Milestone Achieved:* We will determine which cytokines are secreted, by both human and mouse skeletal muscle myocytes, subsequent to entry of DNA into the cytosol**	
Specific Aim 3: Ascertain which components of the human and mouse myocytes' cytosolic DNA surveillance systems are engaged and essential for the induction of inflammatory responses	**Year & Quarter (start to finish)**
Major Task 1: *"In vivo" protein-protein & DNA-protein cross-linking pull-down*	
Subtask 1A: i) optimize membrane permeable chemical cross-linker method with **mouse** cells ii) perform co-immunoprecipitation (co-IP) assays to pull-down proteins complexes formed in DNA exposed vs. non-exposed myocytes iii) perform "time lapse" experiments	**2017 Q1 – 2018 Q1**

Subtask 1B: i) optimize membrane permeable chemical cross-linker method with **human** cells ii) perform co-immunoprecipitation (co-IP) assays to pull-down proteins complexes formed in DNA exposed vs. non-exposed myocytes iii) perform "time lapse" experiments	**2017 Q4 – 2018 Q1**
Subtask 2A: i) label plasmid DNA with photoactivatable biotin (e.g. Psoralen-PEG3-Biotin) ii) perform DNA-protein complex pull-down method to pull-down DNA-protein complexes formed in DNA exposed vs. non-exposed **mouse** myocytes	**2017 Q1 – 2018 Q1**
Subtask 2B: i) label plasmid DNA with photoactivatable biotin (e.g. Psoralen-PEG3-Biotin) ii) perform DNA-protein complex pull-down method to pull-down DNA-protein complexes formed in DNA exposed vs. non-exposed **human** myocytes	**2017 Q4 – 2018 Q1**
Subtask 3A: i) use "shotgun" proteomics methods (solubilized and denatured proteins are separated, subjected to tandem mass spectrometry (MS) and analyzed for identification) to determine the constituents of protein-protein and DNA-protein complexes derived from **mouse** cells	**2017 Q4 – 2018 Q1**
Subtask 3B: i) use "shotgun" proteomics methods (solubilized and denatured proteins are separated, subjected to tandem mass spectrometry (MS) and analyzed for identification) to determine the constituents of protein-protein and DNA-protein complexes derived from **human** cells	**2017 Q4 – 2018 Q1**
Subtask 4A: i) confirm specific **mouse** cell findings using tandem affinity tag purification method whereby candidate partners are co-expressed as 3X FLAG and Twin-StrepTag fusion proteins in **mouse** cells and subsequently subjected to bead-based affinity purification and immunoblotting or ELISA based detection	**2017 Q4 – 2018 Q1**
Subtask 4B: i) confirm specific **human** cell findings using tandem affinity tag purification method whereby candidate partners are co-expressed as 3X FLAG and Twin-StrepTag fusion proteins in **human** cells and subsequently subjected to bead-based affinity purification and immunoblotting or ELISA based detection	**2017 Q4 – 2018 Q1**
***Milestone Achieved:* We will discover which cytosolic DNA sensors/adaptors and downstream effectors are operational in human and mouse skeletal muscle myocytes**	

Major Task 2: *<u>Genome Engineering of Myocyte Cell Lines by CRISPR</u>*	
Subtask 1A: i) design and produce mammalian expression plasmids encoding the Cas9 protein and specially designed gRNAs (that directs the nuclease to complementary **<u>mouse</u>** genomic target sequences) ii) confirm Cas9 and green fluorescent protein (GFP) reporter production by plasmids	**2017 Q4 – 2018 Q1**
Subtask 1B: i) design and produce mammalian expression plasmids encoding the Cas9 protein and specially designed gRNAs (that directs the nuclease to complementary **<u>human</u>** genomic target sequences) ii) confirm Cas9 and green fluorescent protein (GFP) reporter production by plasmids	**2017 Q4 – 2018 Q1**
Subtask 2A: i) produce **<u>mouse</u>** myocyte cell lines that have undergone gene disruption via a technique named CRISPR ii) confirm gene disruption and GFP expression iii) select multiple individual clones characterized with quantitative gene expression	**2017 Q4 – 2018 Q1**
Subtask 2B: i) produce **<u>human</u>** myocyte cell lines that have undergone gene disruption via a technique named CRISPR ii) confirm gene disruption and GFP expression iii) select multiple individual clones characterized with quantitative gene expression	**2017 Q4 – 2018 Q1**
Subtask 3A: i) evaluate **<u>mouse</u>** cell line phenotype upon exposure to cytosolic DNA, as assessed by secretome and transcription factor analyses as described in Specific Aim 2, using cell lines produced with confirmed loss of the genes of interest	**2017 Q4 – 2018 Q1**
Subtask 3B: i) evaluate **<u>human</u>** cell line phenotype upon exposure to cytosolic DNA, as assessed by secretome and transcription factor analyses as described in Specific Aim 2, using cell lines produced with confirmed loss of the genes of interest	**2017 Q4 – 2018 Q1**
<u>Milestone Achieved:</u> We will discover which cytosolic DNA sensors/adaptors and downstream effectors are essential for responding to cytosolic DNA in human and mouse skeletal muscle myocytes	

Cytosolic DNA sensing in skeletal muscle-derived myocytes

Sonia Edayé[1,2] Romina Gheorghe[1] and Gerasimos J. Zaharatos[1,3]

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ABSTRACT

Background: A growing number of cytosolic DNA sensing pathways have been characterized with a role in pathogen detection and activation of host immune defences. In one such pathway, the presence of DNA inside the cytosol induces cGAS (cyclic-GMP-AMP synthase) to activate STING (stimulator of interferon genes), culminating in the transcription of type I interferons and inflammatory cytokines. These pathways have been predominantly characterized using immune cells, such as dendritic cells and macrophages. Gene-based vaccine platforms like DNA vaccines represent a promising approach to prevent infectious diseases. Such vaccines act by delivering a pathogen-derived antigen of interest, encoded by a vector, to host somatic and immune cells. In the case of DNA vaccines, plasmid DNA is generally delivered intramuscularly and thereafter locally transfects skeletal muscle myocytes. The presence of a cytosolic DNA sensing pathway has not been described before in skeletal muscle myocytes and the role of such a pathway in DNA vaccine immunogenicity has not been defined.

Hypothesis and Objective: We hypothesized that cytosolic DNA sensing pathway components, including cGAS and STING, are present in skeletal muscle myocytes and are activated upon entry of DNA into the cytosol. We sought to define the presence and activity of this pathway in a skeletal muscle-derived myocyte model.

Methods: Experiments utilized the mouse skeletal muscle myoblast cell line C2C12. We ascertained the presence of cytosolic DNA sensor pathway constituents using immunoblotting. We then introduced a DNA vaccine vector (a plasmid DNA-based mammalian expression vector expressing GFP) into C2C12 myoblasts or myotubes by chemical transfection methods. The abundance and activation of various DNA sensors and downstream pathways was subsequently characterized by immunoblotting and RT-PCR.

Results: C2C12 myoblasts and myotubes express cytosolic DNA sensor proteins ifi204, cGAS and STING. Introduction of DNA into the myocyte cytosol by transfection results in the phosphorylation of STING. Thereafter, the abundance of STING at the protein level decreases markedly between 2h to 6h post-transfection. Treatment of myoblast cells with MG132, a proteasome inhibitor, partially rescues STING thus suggesting inhibition of degradation. Other downstream components of the DNA sensing pathway such as TBK1 (Tank-binding kinase) and IRF3 (Interferon regulatory factor 3) are also expressed in C2C12 myocytes. The abundance of phosphorylated forms of these proteins (P-TBK1 and P-IRF3) increases after transfection, further suggesting activation of the STING pathway. Finally, transfection results in marked up-regulation of IFN-β1 and IL-6 at the RNA level, again supporting the presence of a functional cytosolic DNA sensing pathway in C2C12.

Conclusion: Taking together our results suggest that skeletal muscle-derived myocytes can sense cytosolic DNA through a STING pathway and that this culminates in the expression of type I interferons and inflammatory cytokines. These findings provide a rationale for further investigation of cytosolic DNA sensing in skeletal muscle and suggest the need for careful consideration of how such events may influence the immunogenicity of intramuscular gene-based vaccine delivery.

ACKNOWLEDGEMENTS

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BACKGROUND

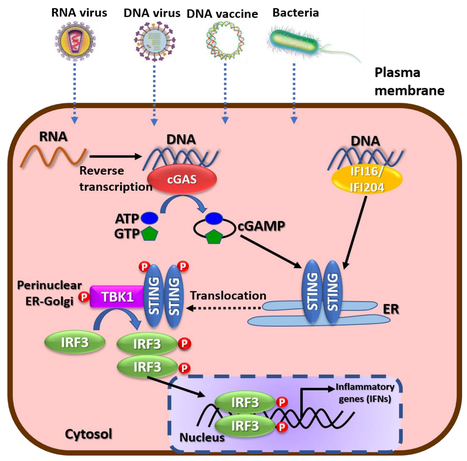

Figure 1. Cytosolic DNA sensing cGAS-STING pathway.
After recognition of cytosolic DNA, cGAS catalyzes the synthesis of cGAMP which acts as a second messenger and binds to the adaptor protein STING. IFI16/IFI204 also recognizes cytosolic DNA and activates STING. STING dimerizes and translocates to a perinuclear region (ER-Golgi). Following STING relocalization, TBK-1 is recruited to STING. After binding to STING, TBK1 undergoes self-phosphorylation and subsequently phosphorylate STING and IRF-3. IRF3 then translocate into the nucleus and induces the production of type I IFN.

RESULTS 1

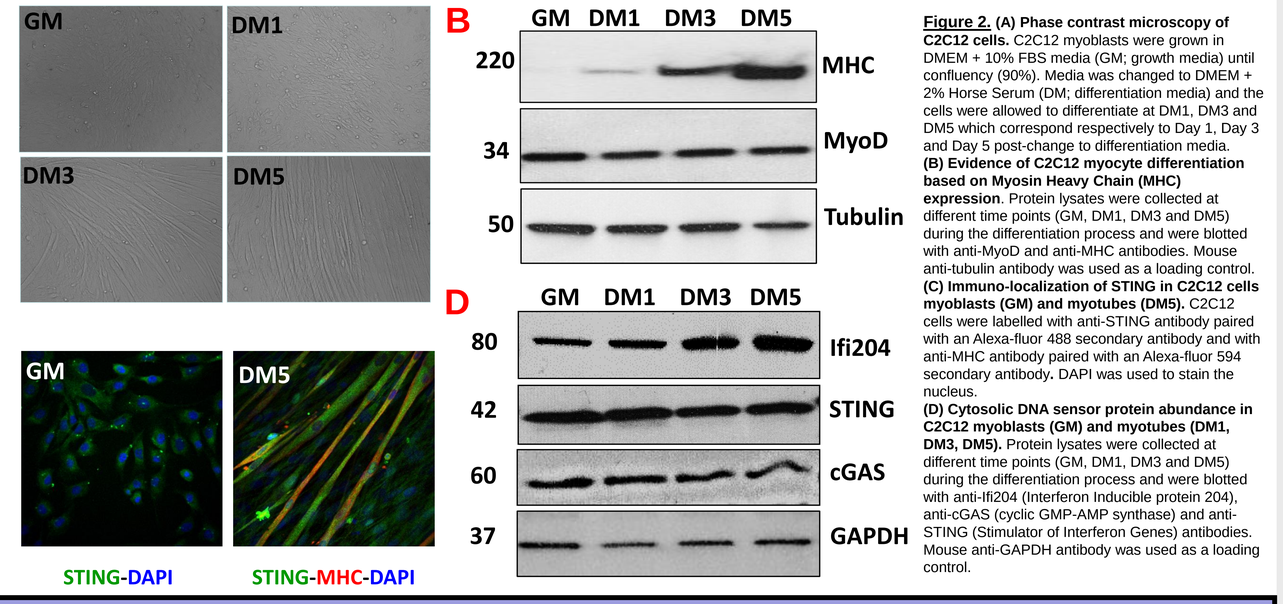

Figure 2. (A) Phase contrast microscopy of C2C12 cells. C2C12 myoblasts were grown in DMEM + 10% FBS media (GM; growth media) until confluency (90%). Media was changed to DMEM + 2% Horse Serum (DM; differentiation media) and the cells were allowed to differentiate at DM1, DM3 and DM5 which correspond respectively to Day 1, Day 3 and Day 5 post-change to differentiation media.
(B) Evidence of C2C12 myocyte differentiation based on Myosin Heavy Chain (MHC) expression. Protein lysates were collected at different time points (GM, DM1, DM3 and DM5) during the differentiation process and were blotted with anti-MyoD and anti-MHC antibodies. Mouse anti-tubulin antibody was used as a loading control.
(C) Immuno-localization of STING in C2C12 cells myoblasts (GM) and myotubes (DM5). C2C12 cells were labelled with anti-STING antibody paired with an Alexa-fluor 488 secondary antibody and with anti-MHC antibody paired with an Alexa-fluor 594 secondary antibody**.** DAPI was used to stain the nucleus.
(D) Cytosolic DNA sensor protein abundance in C2C12 myoblasts (GM) and myotubes (DM1, DM3, DM5). Protein lysates were collected at different time points (GM, DM1, DM3 and DM5) during the differentiation process and were blotted with anti-Ifi204 (Interferon Inducible protein 204), anti-cGAS (cyclic GMP-AMP synthase) and anti-STING (Stimulator of Interferon Genes) antibodies. Mouse anti-GAPDH antibody was used as a loading control.

RESULTS 2

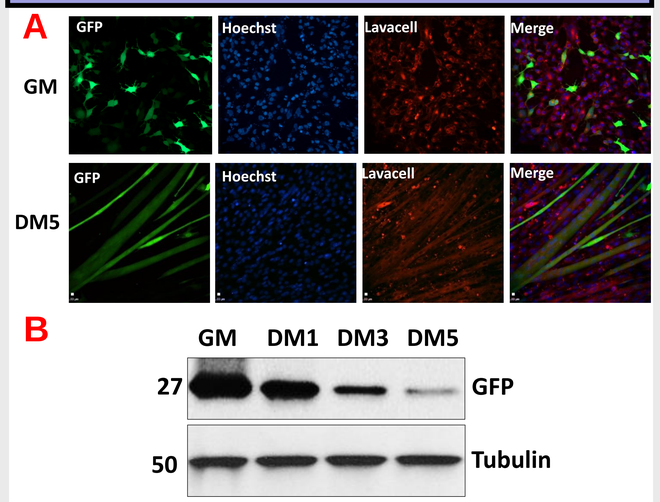

Figure 3. (A) Confocal microscopy of C2C12 pVAX-GFP transfected cells. C2C12 myoblasts were transfected with pVAX-GFP and 48h after transfection (GM) the transfected cells were allowed to differentiate for 5 days (DM5). GFP fluorescence of the cells was imaged using excitation with a 488 nm laser. Hoechst dye (excited with 405 nm laser) was used to stain the nucleus while Lavacell (Active Motif) was used to label membrane proteins (excited with 561 nm laser).
(B) GFP expression after C2C12 cells transfection. Protein lysates were collected at different time points (GM, DM1, DM3 and DM5) after transfection with pVAX-GFP and were blotted with anti-GFP antibody. Mouse anti-tubulin antibody was used as a loading control.

RESULTS 3

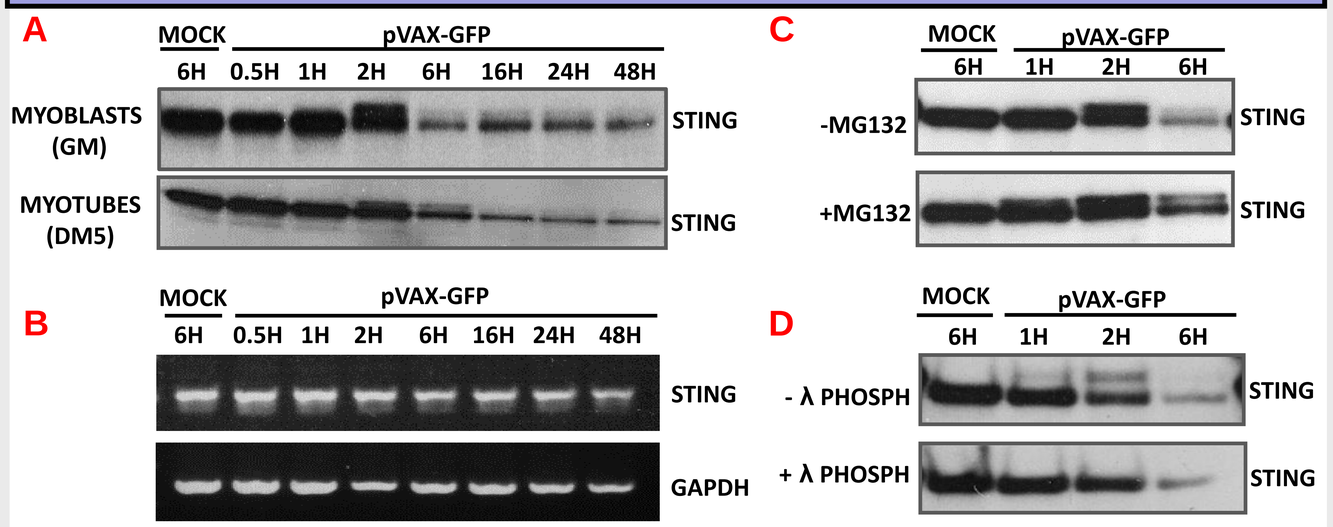

Figure 4. (A) STING abundance is diminished between 2h and 6h after transfection of C2C12 cells with pVAX-GFP. Protein lysates from myoblast (GM) or myotubes (DM5) were collected 6h after mock transfection without DNA (Mock) or 0.5h, 1h, 2h, 6h, 16h, 24h and 48h after transfection with pVAX-GFP and were blotted with anti-STING antibody.
(B) Loss of STING doesn't occur at the RNA level. RT-PCR analysis was performed using total RNA extracts from myoblasts collected 6h after mock transfection without DNA (Mock) or 0.5h, 1h, 2h, 6h, 16h, 24h and 48h after transfection with pVAX-GFP. Mouse-STING specific primers were used to amplify STING and Mouse-GAPDH primers were used for GAPDH as an internal control.
(C) STING appears to be degraded through the proteasome pathway. Cells underwent treatment with 400μM MG132 or no treatment prior to transfection. Protein lysates from myoblasts were collected 6h after mock transfection without DNA (Mock) or 1h, 2h and 6h after transfection with pVAX-GFP. The lysates were blotted with anti-STING antibody.
(D) STING is phosphorylated prior to degradation. Protein lysates from myoblasts were collected 6h after mock transfection without DNA (Mock) or 1h, 2h and 6h after transfection with pVAX-GFP. The lysates underwent treatment with λ Phosphatase (100U) or no treatment and were then blotted with anti-STING antibody.

RESULTS 4

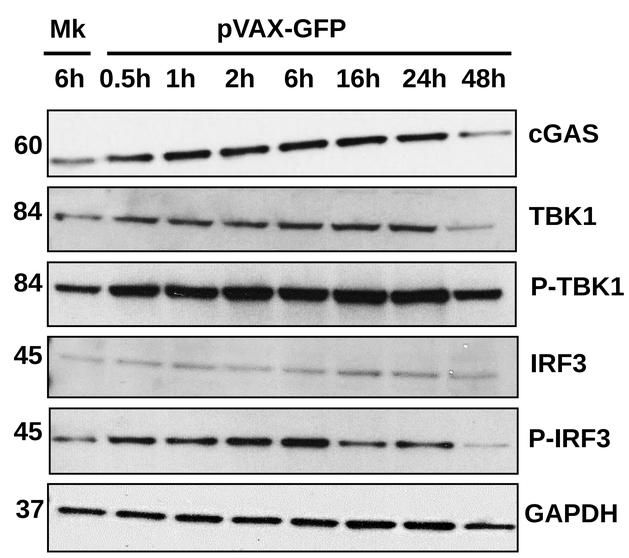

Figure 5. Kinetics of cytosolic DNA sensor protein expression. Protein lysates were collected 6h after mock transfection without DNA (Mk) or 0.5h, 1h, 2h, 6h, 16h, 24h and 48h after transfection with pVAX-GFP and were then blotted with anti-STING, anti-cGAS, anti-TBK1, anti-P-TBK1, anti-IRF3 and anti-P-IRF3 antibodies. Mouse GAPDH antibody was used as a loading control.

RESULTS 5

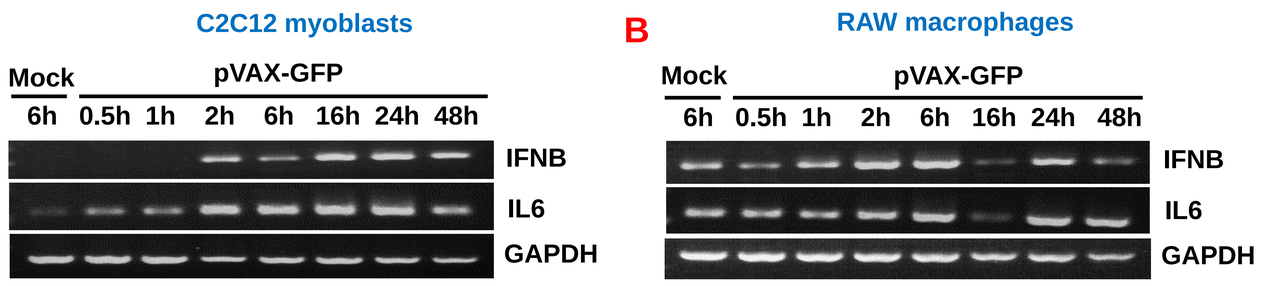

Figure 6. (A) IFNB and IL6 expression in C2C12 cells. RT-PCR analysis was performed using total RNA extracts from myoblast cells collected 6h after mock transfection without DNA (Mock) or 0.5h, 1h, 2h, 6h, 16h, 24h and 48h after transfection with pVAX-GFP. Mouse-IFNβ1 and mouse-IL6 specific primers were used to amplify IFNβ1 and IL6 respectively. Mouse-GAPDH primers were used for GAPDH as an internal control.
(B) IFNB and IL6 expression in RAW cells. RT-PCR analysis was performed using total RNA extracts from macrophages cells collected 6h after mock transfection without DNA (Mock) or 0.5h, 1h, 2h, 6h, 16h, 24h and 48h after transfection with pVAX-GFP. Mouse-IFNβ1 and mouse-IL6 specific primers were used to amplify IFNβ1 and IL6 respectively. Mouse-GAPDH primers were used for GAPDH as an internal control.

CONCLUSION AND FUTURE DIRECTIONS

- Skeletal muscle myocytes can sense cytosolic DNA and appear to do so through STING
- This pathway can trigger expression of type I interferons and inflammatory cytokines
- Our findings provide a rationale for further investigation of cytosolic DNA sensing in skeletal muscle
- We are endeavoring to better delineate the components of the cytosolic DNA sensing pathway that are engaged in myoblasts and myotubes
- The relevance of these events will be explored in small animal models

Are myocytes actors in DNA vaccine immunogenicity? *In vitro* studies of cytosolic DNA sensing in skeletal muscle myocytes

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Background: DNA vaccines and other gene-based vaccine platforms represent a promising approach to prevent infectious diseases. In the case of DNA vaccines, plasmid DNA is generally delivered intramuscularly and thereafter locally transfects skeletal muscle myocytes. Cytosolic DNA sensing pathways have predominantly been characterized using immune cells but have not been described in skeletal muscle myocytes. As such the impact of myocyte sensing of DNA on DNA vaccine immunogenicity has not been defined. **Hypothesis:** We hypothesized that cytosolic DNA sensing pathway components are present in skeletal muscle myocytes and are activated upon entry of DNA into the cytosol to initiate a pro-inflammatory response. **Methods:** We introduced a DNA vaccine vector into C2C12 myoblasts or myotubes by chemical transfection methods. The presence, abundance and activation of various DNA sensors and downstream pathways was characterized by immunoblotting, biochemical and RT-PCR methods. **Results:** C2C12 myoblasts and myotubes express cytosolic DNA sensor proteins cGAS and STING. The transfection of DNA into C2C12 results in the phosphorylation of STING and other downstream components of the DNA sensing pathway such as TBK1 and IRF3. The abundance of phosphorylated forms of these proteins increases after transfection followed by the up-regulation of IFN-β1 and IL-6 at the RNA level, supporting the presence of a functional cytosolic DNA sensing pathway in C2C12. **Conclusion:** Taking together our results suggest that skeletal muscle-derived myocytes can sense cytosolic DNA, thus providing a rationale for further investigation of cytosolic DNA sensing in skeletal muscle. Our findings suggest the need to carefully consider how such events might influence the immunogenicity of gene-based vaccines delivered via the intramuscular route.